

THE TRANSFORMATION OF PNEUMOCOCCAL TYPES

I. THE CONVERSION OF R FORMS OF PNEUMOCOCCUS INTO S FORMS OF THE HOMOLOGOUS TYPE*

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In previous communications (1, 2) it was shown that avirulent, non-type-specific R forms of *Pneumococcus* may be converted into virulent, type-specific, S organisms either "*in vivo*" by animal passage, or "*in vitro*" by growth in anti-R serum. Conversion by both methods was invariably accompanied by the acquisition of all the characteristics of the S type, including maximal virulence. In every instance in which the change was effected by these procedures the R forms were converted to the same specific type from which they were originally derived.

Since the publication of the foregoing results there has appeared a most significant article by F. Griffith (3), in which he stated that:

1) R forms of *Pneumococcus* may be converted into S forms of the homologous type by the subcutaneous injection, in white mice, of large amounts of living R organisms.

2) R forms of *Pneumococcus* may be similarly converted into S forms of the homologous type by the subcutaneous injection, in white mice, of small amounts of living R organisms together with the heat-killed bacteria from large amounts of homologous S cultures.

3) R forms of *Pneumococcus* may be transformed into S forms of heterologous types by the subcutaneous injection, in white mice, of small amounts of living R organisms together with the heat-killed bacteria from large amounts of heterologous S cultures.

The present communication is concerned with the first two of the

* In this and succeeding instances 'homologous type' indicates that specific S type from which the R forms were originally derived.

above findings. The third finding, which involves the question of actual transformation of type, is the subject of the succeeding paper.

A. Conversion of R Forms of Pneumococcus into S Forms of the Homologous Type by the Subcutaneous Injection, in White Mice, of Large Amounts of Living R Organisms

Methods

Ten-hour plain broth cultures of *Pneumococcus* were centrifuged and the bacteria were resuspended in plain broth in varying dilutions as outlined in the following experiments. Mice were inoculated subcutaneously in the right inguinal region, with 0.5 cc. of each of the various dilutions, care being taken that none of the material escaped along the track of the needle. All mice were autopsied in the following manner: The skin was washed with alcohol and a subcutaneous incision was made along the mid-line of the abdomen. A flap of skin was then reflected and cultures were made from the site of injection on blood agar plates and in blood broth. The inguinal gland, or a portion of tissue from this region, was carefully excised under sterile precautions and cultures were made from this material in blood broth. Except in earlier experiments contaminations rarely occurred. Occasionally mice developed ulcers at the site of injection and such animals were discarded. Cultures were made from the heart's blood in the usual manner.

EXPERIMENTAL

(a) 2 R culture, strain D/39/R.

This culture was obtained from a typical Type II S *Pneumococcus* by growth in homologous immune serum. Its virulence was such that 0.5 cc. of culture occasionally killed white mice, but amounts of 0.25 cc. or less uniformly failed to do so. It produced only Rough colonies, did not agglutinate specifically in type sera, and did not produce the specific soluble substance upon which type-specificity depends (4). It could be changed to the S type by either the "*in vivo*" method of animal passage, or by the "*in vitro*" method of growth in media containing anti-R serum. Three to four mouse passages usually sufficed to bring about the R→S change, while five to seven transfers in 10 per cent anti-R serum induced a similar transformation. Single-cell cultures derived from the mass culture have been shown to react in precisely the same manner.

A series of eight mice were injected subcutaneously in the right inguinal region with the bacteria from varying amounts of culture, a volume of 0.5 cc. being injected in each instance.

TABLE I

No mice	Amount of living R forms injected—in terms of original culture	Result	Kinds of colonies recovered in cultures		Agglutination H.B. culture
			P.I.	H.B.	
2	10	1 d. 40 hrs.	R and S	S	Type II
		2 " " "	" " "	"	" "
2	5	1 d. 18 hrs.	R only	R only	R
		2 " 40 "	R and S	S	Type II
2	2.5	1 d. 40 hrs.	" " "	"	" "
		2 " 72 "	" " "	"	" "
2	1	1 d. 30 hrs.	" " "	"	" "
		2 " 48 "	" " "	"	" "

d; died.

P. I. place of injection.

H. B. heart's blood.

Summary: No. mice injected, 8.

Died, 8. Reversion to the homologous S type, 7.

No reversions, only R organisms recovered, 1.

In seven out of eight animals injected, type-specific organisms, possessing all the attributes of the S type, were recovered from the heart's blood. Cultures from the site of injection yielded a mixture of R and S colonies. The one animal yielding only R forms died in 18 hours, apparently before there was time for the transformation to be effected.

(b) 3 R culture, strain M/3/R.

This culture was obtained from a typical Type III S Pneumococcus by growth in homologous immune serum. It possessed all the characteristics of the R form and could be converted to the S type, although not so readily as the 2 R culture above described. Twenty to thirty animal passages by the intraperitoneal route were necessary to restore type-specificity, while ten to fifteen transfers in 10 per cent anti-R serum were required to effect the R→S change.

Ten mice were injected subcutaneously as follows:—

TABLE II

No. mice	Amount of living R forms injected—in terms of original culture	Result	Kinds of colonies recovered in cultures		Agglutination H.B. culture
			P.I.	H.B.	
2	30	1 d. 18 hrs.	R only	R only	R
		2 " 48 "	R and S	S	Type III
2	15	1 d. 48 hrs.	R and S	S	" "
		2 " 64 "	" " "	S	" "
2	10	1 d. 18 hrs.	R only	R only	R
		2 " 100 "	R and S	S	Type III
2	5	1 d. 40 hrs.	R and S	S	" "
		2 s.	—	—	—
2	2.5	1 s.	—	—	—
		2 s.	—	—	—

d; died.

s; survived.

P. I. place of injection.

H. B. heart's blood.

Summary: No. mice injected, 10.

Died, 7. Reversion to the homologous S type, 5.

No reversion, only R organisms recovered, 2.

Survived, 3.

While this culture reverted to the homologous S type in five out of ten animals injected, larger doses were required than with the 2 R culture described. This finding is in accord with the results obtained by the other methods of inducing reversion.

(c) 1 R culture, strain 1/192/R.

This R culture had been under artificial cultivation in this laboratory for many years. All previous efforts to effect reversion to the S type had been unsuccessful. Reimann (5) passed the same strain through 105 consecutive mice without altering its characteristics and 100 transfers in 10 per cent anti-R serum likewise induced no change.

Nine mice were injected subcutaneously, each with the bacteria from 50 cc. of R culture, as follows:

TABLE III

No. mice	Amount of living R forms injected—in terms of original culture	Result	Kinds of colonies recovered in cultures		Agglutination H.B. culture
			P.I.	H.B.	
9	50	1 d. 18 hrs.	R only	R only	R
		2 " " "	" "	" "	"
		3 " " "	" "	" "	"
		4 " " "	" "	" "	"
		5 " 21 "	" "	" "	"
		6 " " "	" "	" "	"
		7 " 60 "	" "	" "	"
		8 " 80 "	" "	" "	"
		9 s.	—	—	—

d; died.

s; survived.

P. I. place of injection.

H. B. heart's blood.

Summary: No mice injected, 9.

Died, 8. Reversion to the homologous type, O.

No reversion, only R organisms recovered 8.

Survived, 1.

In spite of the large amounts of organisms employed,—the bacteria from 50 cc. of culture—this strain, in all animals, failed to revert to the S type. Smaller amounts of culture were used in other experiments with uniformly negative results. This finding offers further evidence of the existence of different degrees of constancy in the R form of *Pneumococcus* and confirms the results previously obtained.

Conversion of Single-Cell R Cultures into S Forms of the Homologous Type

In a previous paper (2) it was reported that single-cell cultures have always reacted in the same manner as the mass cultures from which they were obtained. To substantiate this finding single-cell R strains were selected from the above mass cultures, according to the method of Avery and Leland (6), and injected subcutaneously into white mice. In all cases essentially the same results were obtained as when the

mass cultures were used. The following protocol shows the results of a typical experiment:

Single-cell strain, 2 R culture, strain D/39/R.

TABLE IV

No. mice	Amount of living R forms injected—in terms of original culture	Result	Kinds of colonies recovered in cultures		Agglutination H.B. culture
			P.I.	H.B.	
2	10	1 d. 24 hrs. 2 " 40 "	R only R and S	R only S	R Type II
2	5	1 d. 22 hrs. 2 " 24 "	R only " "	R only " "	R R
2	2.5	1 d. 54 hrs. 2 s.	R and S —	S —	Type II —
2	1	1 d. 40 hrs. 2 s.	R and S —	S —	Type II —

d; died.

s; survived.

P. I. place of injection.

H. B. heart's blood.

Summary: No. mice injected, 8.

Died, 6. Reversion to the homologous S type, 3.

No reversion, only R organisms recovered, 3.

Survived, 2.

In this experiment three mice apparently died prematurely of an R infection. Otherwise the results were essentially the same as those recorded in Table I in which the mass culture was employed.

Attempts to Cause Further "Degradation" of an R Culture by Prolonged Growth in Homologous Immune Serum

In the course of later work it became essential to have a 2 R culture which would not revert so readily to the S type. Accordingly the above 2 R strain was grown in 50 per cent Type II serum for twelve further transfers and the resulting culture injected into mice as follows:—

TABLE V

No. mice	Amount of living R forms injected—in terms of original culture	Result	Kinds of colonies recovered in cultures		Agglutination H.B. culture
			P.I.	H.B.	
2	10	1 d. 21 hrs.	R only	R only	R
		2 " 40 "	R and S	S	Type II
2	5	1 d. 40 hrs.	R and S	S	Type II
		2 " " "	" " "	"	" "
2	2.5	1 d. 54 hrs.	R and S	S	Type II
		2 s.	—	—	—
2	1	1 d. 70 hrs.	R and S	S	Type II
		2 s.	—	—	—

d; died.

s; survived.

P. I. place of injection.

H. B. heart's blood.

Summary: No. mice injected, 8.

Died, 6. Reversion to the homologous S type, 5.

No reversion, only R organisms recovered, 1.

Survived, 2.

By comparison with the results in Table IV it is seen that, in spite of twelve further transfers in 50 per cent homologous immune serum, this strain reverted to the homologous S type as readily as the original culture.

It would appear that an R culture possesses the capacity to become "stabilized" at some phase of the "degradation" process. In subsequent experiments evidence will be offered to show that a corresponding condition of partial degradation may occur in S cultures. In other words, the terms R and S, as applied to cultures of *Pneumococcus*, have only a relative value.

The results of the preceding experiments may be summarized as follows:—R forms of *Pneumococcus* can be converted into S forms of the homologous type by the subcutaneous injection in white mice, of suitable amounts of R cultures. As in other methods, conversion was

invariably accompanied by the acquisition of all the attributes of the S type, including maximal virulence. Single-cell cultures reacted in the same manner as the mass cultures from which they were derived. Attempts to cause a further "degradation" of R forms by continued growth in homologous immune serum were unsuccessful.

B. Conversion of R Forms of Pneumococci into S Forms of the Homologous Type by the Subcutaneous Injection, in White Mice, of Small Amounts of Living R Organisms Together with the Heat-Killed Bacteria from Large Amounts of the Homologous "S" Culture

Methods

It is of paramount importance to consider in detail the methods employed in the production of the vaccines¹ and the controls adopted to eliminate the possibility of the persistence of viable forms in the suspensions of heat-killed organisms. All vaccines were made from 1500 cc. of plain broth cultures grown in 3 liter flasks. In earlier experiments little attention was paid to the phase of growth at which the culture was killed, or to the amount of autolysis which might have taken place at the time of heating. In later experiments, however, these factors were found to be of considerable importance, and ten-hour plain broth cultures were uniformly used. Moderately heavy growth was found to be essential. After ten hours growth at 37°C. the flasks of culture were subjected to a *preliminary* heating at 60° for 10 minutes. This preliminary heating inhibited further autolysis and was found to have considerable influence on the efficacy of the vaccine. The cultures were concentrated by centrifuging and taken up in 1/100th of their original volume of plain broth. This concentrated material was transferred to glass ampules, sealed in a blow flame, and heated when totally immersed in water. The vaccines were subjected to definite temperatures for varying periods, as will later be described. Since the factors of time and heat materially altered the results obtained they were most carefully regulated. Fifteen minutes was the shortest period and 60°C. the lowest temperature to which the bacterial suspensions were exposed, and invariably this minimal exposure was found sufficient to kill all pneumococci. In many experiments, however, the vaccines were heated at much higher temperatures and for longer periods of time.

Mice were injected subcutaneously in the right inguinal region, the total volume introduced never exceeding 0.75 cc. Under these conditions the development of ulcers at the site of injection did not occur except in occasional instances. The

¹ For the sake of convenience the term vaccine is used to denote a suspension of heat-killed organisms.

animals were autopsied as described in the first part of this paper. Contaminations were rarely encountered. Cultures were invariably made, both from the site of injection and the heart's blood, on blood agar plates and in blood broth. The colonies were examined under a Zeiss "plate culture" microscope; but morphology alone was never considered a final criterion as to the nature of the organisms constituting the colony. Agglutination tests were done on all cultures. In cases of doubt a second mouse was inoculated and the organisms from the peritoneal contents were typed in the usual manner.

Controls on the Viability of the Vaccines:

The possibility of potentially viable organisms surviving in the concentrated vaccines demanded that more than ordinary control measures should be adopted to eliminate such a contingency. The "*in vitro*" and "*in vivo*" controls employed were as follows:

1. *In Vitro* Controls:

(a) Cultures were made from all vaccines in blood broth and on blood agar plates. In many experiments this was done in varying dilutions. In no instance was growth obtained.

(b) Many lots of S vaccine were used repeatedly in "*in vitro*" attempts to secure the R→S change. Broth containing concentrated S vaccine was seeded with R forms and subcultured serially for twenty transfers. No growth of "S" organisms occurred and the final culture remained avirulent for mice.

(c) In one critical experiment cultures were made, both aerobically and anaerobically, in 5 per cent blood broth and blood-extract dextrose broth. The cultures were incubated two weeks, plates poured, and the material injected into mice. No growth occurred and the mice survived.

2. "*In Vivo*" Controls:

(a) Control mice were injected with the vaccine alone. At least four mice were always used and in many experiments the number of control animals was equal to the number of experimental animals. Varying amounts of vaccine up to and including the bacteria from 100 cc. of culture were injected. Both the subcutaneous and intraperitoneal routes were used. Without exception all animals survived. They were sacrificed at intervals up to three weeks and autopsied. The inguinal lymph gland, or a portion of subcutaneous tissue at the site of injection, was dissected out and cultures were made from this material in blood broth and on blood agar plates. In some cases this tissue was ground up and injected into other mice. Cultures were also invariably made from the heart's blood. In no instance were living pneumococci recovered.

(b) Control mice were injected with the vaccine together with other live organisms. The possibility of the existence in the vaccines of potentially viable organisms, which could not multiply by themselves, but which might, in some way, be stimulated to renewed growth by other live organisms, received careful consideration. Mice were injected with vaccines together with living cultures of *Staphylococcus*, *Streptococcus*, *B. Influenzae*, and Friedländer's bacillus. All

animals which succumbed were autopsied and all surviving animals were sacrificed at appropriate intervals and careful cultures were made. In no instance was a viable pneumococcus recovered.

As Griffith pointed out (3), in certain instances, the temperature at which the vaccines were heated exerted a definite influence on the effect which they produced. The following experiments are therefore divided into two groups, 1) those in which the vaccines were heated at 60°C, and 2) those in which the vaccines were heated at 100°C. In both groups the time of heating was fifteen minutes.

I. R Cultures Together with Homologous S Vaccines, Heated for 15' at 60°C.

(a) 1 R Culture (Strain 1/192/R) + 1 S Vaccine, Heated for 15' at 60°C.

TABLE VI

No. mice	Amount of culture from which heat-killed organisms were obtained	Amt. of living R culture	Result	Kinds of colonies recovered in cultures		Agglutination H.B. culture
				P.I.	H.B.	
4 Controls	cc. 90	cc. Nil.	All survived. Sacrificed 7 days	All cultures negative		
6	90	0.25	1 d. 1½ days 2 " " " 3 " 5 " 4 " 7 " 5 s. k. 7 " 6 s. k. 7 "	R only R and S S S R only R only	R only S S S — —	R Type I " " " " — —

d; died.

s; survived.

k; killed.

P. I. place of injection.

H. B. heart's blood.

Summary: No. test mice injected, 6.
 Died, 4. Reversion to the homologous S type, 3.
 No reversion, only R organisms recovered, 1.
 Survived, 2. R organisms recovered when sacrificed, 2.

(b) 2 R Culture (Strain D/39/R) + II S Vaccine, Heated for 15' at 60°C.

TABLE VII

No. mice	Amount of culture from which heat-killed organisms were obtained	Amt. of living R culture	Result	Kinds of colonies recovered in colonies		Agglutination H.B. culture
				P.I.	H.B.	
4 Controls	cc. 90	cc. Nil.	All survived. Sacrificed 7 days	All cultures	neg- ative	
6	90	0.25	1 d. 1½ days	R and S	S	Type II
			2 " " "	" " "	S	" "
			3 " 2 "	" " "	S	" "
			4 " " "	" " "	S	" "
			5 " " "	" " "	S	" "
			6 s. ulcer at P.I.	—	—	—

d; died.

s; survived.

P. I. place of injection.

H. B. heart's blood.

Summary: No. test mice injected, 6.
 Died, 5. Reversion to the homologous S type, 5.
 Survived, 1. (Ulcer).

(c) 3 R Culture (Strain M/3/R) + III S Vaccine, Heated for 15' at 60°C

TABLE VIII

No. mice	Amount of culture from which heat-killed organisms were obtained	Amt. of living R culture	Result	Kinds of colonies recovered in colonies		Agglutination H.B. culture
				P.I.	H.B.	
4 Controls	90	Nil.	All survived. Sacrificed 7 days	All cultures	negative	
6	90	0.25	1 d. 2 days	R and S	S	Type III
			2 " " "	" " "	"	" "
			3 " 2½ "	" " "	"	" "
			4 " 3 "	" " "	"	" "
			5 " 4 "	" " "	"	" "
			6 s. k. 11 "	R only	—	—

d; died.

s; survived.

k; killed.

P. I. place of injection.

H. B. heart's blood.

Summary: No. test mice injected, 6.

Died, 5. Reversion to the homologous S type, 5.

Survived, 1. R organisms recovered when sacrificed, 1.

The results of the three preceding experiments may be summarized as follows; R forms of Pneumococcus, when injected subcutaneously in white mice, together with S vaccines of the homologous type, were converted, in the majority of the animals, to the specific S type from which they were originally derived. In these experiments the vaccines were heated for 15' at 60°C. All control mice survived. At the end of seven days the controls were sacrificed and cultures were made from both the site of injection and the heart's blood. Without exception all cultures were sterile. The 1 R culture (Strain 1/192/R) which had remained totally avirulent after all previous efforts to effect the R→S change, reverted to the S type in three out of six animals injected.

II. R Cultures Together with Homologous S Vaccines, Heated for 15' at 100°C.

(a) 1 R Culture (Strain 1/192/R) + 1 S Vaccine, Heated for 15' at 100°C.

TABLE IX

No. mice	Amount of culture from which heat-killed organisms were obtained	Amt. of living R culture	Result	Kinds of colonies recovered in cultures		Agglutination H.B. culture
				P.I.	H.B.	
4 Controls	cc. 90	cc. Nil.	All survived. Sacrificed 7 days	All cultures negative		
6	90	0.25	1 d. 1½ days	R only	R only	R
			2 " 2 "	R only	R only	R
			3 s.)	—	—	—
			4 s.)	—	—	—
			5 s.)	R only	—	—
			6 s.)	—	—	—

d; died.

s; survived.

k; killed.

P. I. place of injection.

H. B. heart's blood.

Summary: No. test mice injected, 6.

Died, 2. Reversion to the homologous S type, 0.

No reversion, only R organisms recovered, 2.

Survived, 4. R organisms recovered when sacrificed, 1.

Since reversion to the S type failed to occur in any of the mice which received Type I S vaccine heated at 100°, while the change was frequently effected in those which received the vaccine heated at 60°C., this experiment was repeated. Entirely similar results were obtained. Apparently, as reported in Griffith's paper, Type I vaccine, heated at 100°C. fails to produce the change brought about by the vaccine heated at 60°C.

Attention is drawn to the fact, that, in some mice, living R forms

were found in the subcutaneous tissues nine days after injection. In other experiments they have been recovered as late as twenty days after inoculation. The ability of R forms to survive in the tissues, then, is not the only condition necessary to bring about conversion to the S type.

(b) 2 R Culture (Strain D/39/R) + II S Vaccine, Heated for 15' at 100°C.

TABLE X

No. mice	Amount of culture from which heat-killed organisms were obtained	Amt. of living R culture	Result	Kinds of colonies recovered in colonies		Agglutination H.B. culture
				P.I.	H.B.	
4 Controls	cc. 90	cc. Nil.	All survived. Sacrificed 9 days	All cultures negative		
6	90	0.25	1 d. 1½ days 2 " " " 3 " 2 " 4 " " " 5 " " " 6 " " "	R and S " " " " " " " " " " " " " " "	S " " " " "	Type II " " " " " " " " " "

d; died.

P. I. place of injection.

H. B. heart's blood.

Summary: No. test mice injected, 6.

Died, 6. Reversion to the homologous S type, 6.

(c) 3 R Culture (Strain M/3/R) + III S Vaccine, Heated for 15' at 100°C.

TABLE XI

No. mice	Amount of culture from which heat-killed organisms were obtained	Amt. of living R culture	Result	Kinds of colonies recovered in colonies		Agglutination R.B. culture
				P.I.	H.B.	
4 Controls	cc. 90	cc. Nil.	All survived. Sacrificed 9 days	All cultures negative		
6	90	0.25	1 d. 1½ days	R and S	S	Type III
			2 " 2 "	" " "	S	" "
			3 " " "	" " "	S	" "
			4 " " "	" " "	S	" "
			5 s. k. 13 "	R only	—	—
			6 s. k. " "	—	—	—

d; died.

s; survived.

k; killed.

P. I. place of injection.

H. B. heart's blood.

Summary: No. test mice injected, 6.

Died, 4. Reversion to the homologous S type, 4.

Survived, 2. R organisms recovered when sacrificed, 1.

The preceding experiments show that vaccines prepared from Types II S and III S organisms are equally effective in producing reversion whether heated for 15 minutes at 60°C. or for 15 minutes at 100°C. Type I S vaccine, on the other hand, while effective in causing reversion of 1 R forms to the homologous S type when heated for 15 minutes at 60°C. did not possess this property when heated for 15 minutes at 100°C.

Two possible explanations may be advanced for the apparent differences in effect produced by Type I S vaccine heated at 60° and at 100°C. First, that property of the vaccine responsible for reversion might have been destroyed, in the case of Type I vaccine, by heating at 100°C. but not in the case of Type II and Type III vaccines similarly

treated. Second, the failure of Type I vaccine to effect reversion when heated at 100°C. might not have been due to the destruction of any property of the vaccine itself, but rather to the difficulty which had always been encountered in effecting the R→S change with this particular 1 R strain. It has been repeatedly shown that the 2 R and 3 R strains employed in these experiments can be much more readily converted to the S type. However, in view of the entirely similar results recorded by Griffith, and because of the findings to be reported in the subsequent paper, the former explanation is much the more probable.

*Attempts to Effect the R→S Change by the Injection of Living R
Organisms Together with the Heat-Killed Bacteria from Large
Amounts of R Cultures*

It was thought that the effect of the vaccines in producing reversion might be due to one of two causes:—First, the injection of such large amounts of heat-killed culture might overwhelm the general resistance of the animal and so allow the R forms to grow in an environment suitable for the development of S types. Second, the vaccines might act locally to protect the R forms from phagocytosis, and so enable them to survive and produce their own S substance. In either case it was thought that the vaccine of an R culture, if injected in sufficiently large amounts together with living R organisms, would similarly allow reversion to take place.

2 R Culture + 2 R Vaccine of the Same Strain Heated for 15' at 60°C. (Strain D/39/R).

TABLE XII

No. mice	Amount of culture from which heat-killed organisms were obtained	Amt. of living R culture	Result	Kinds of colonies recovered in cultures		Agglutination H.B. cultures
				P.I.	H.B.	
2	cc. 200	cc. 0.25	1 d. 2 days 2 s. k. 11 "	R only —	R only —	R —
4	150	0.25	1 d. $\frac{1}{2}$ days 2 " 1 "	R only " "	R only R only (2 colonies)	R R
			3 " $2\frac{1}{2}$ " 4 s. k. 11 "	" " —	R only (few) —	R —
4	100	0.25	1 d. 1 day 2 " $1\frac{1}{2}$ "	R only " "	R only " " (8 colonies)	R R
			3 " " "	" "	R only (6 colonies)	R
			4 " " "	" "	R only	R

d; died.

s; survived.

k; killed.

P. I. place of injection.

H. B. heart's blood.

3 R Culture + 3 R Vaccine of the Same Strain Heated for 15' at 60°C. (Strain M/3/R).

TABLE XIII

No. mice	Amount of culture from which heat-killed organisms were obtained	Amt. of living R culture	Result	Kinds of colonies recovered in cultures		Agglutination H.B. cultures
				P.I.	H.B.	
2	cc. 200	cc. 0.25	1 d. 1 day 2 s. k. 11 "	R only —	R only —	R —
4	150	0.25	1 } 2 } s. k. 11 days 3 } 4 }	— — — —	— — — —	— — — —
4	100	0.25	1 d. $\frac{1}{2}$ " 2 " 1 " 3 } s. k. 11 " 4 }	R only " " — —	R only R only (6 colonies) — —	R R — —

d; died.

s; survived.

k; killed.

P. I. place of injection.

H. B. heart's blood.

Summary: (Tables XII and XIII).

No. test mice injected, 20.

Died, 11. Reversion to the homologous S type, 0.

No reversion, only R organisms recovered, 11.

Survived, 9. R organisms recovered when sacrificed, 0.

R vaccines, even when heated for so short a period as 15 minutes at 60°, and in huge doses, representing the bacteria from 200 cc., 150 cc., and 100 cc. of broth culture, completely failed to cause the R cultures to revert to their own type. Similar negative results were obtained when vaccines of S Friedländer bacilli were inoculated together with the R forms of Pneumococcus. Francis (7), in this laboratory, has made similar observations while working with rabbits. He found that S vaccines were effective in causing R forms to revert to the S type;

while R vaccines and vaccines of *Staphylococcus* failed to produce the change.

"In Vitro" Attempts to Effect the R→S Change by Growth of R Forms with S Cultures and with S Vaccines

(1) In previous work experiments had been done to observe the effect of growing R and S cultures in symbiosis. R and S forms of the same strain were grown together for serial transfers in varying dilutions, as follows:—

	cc.						
Dilution of S Culture.....	10 ⁻⁷	10 ⁻⁶	10 ⁻⁵	10 ⁻⁴	10 ⁻³	10 ⁻²	10 ⁻¹
Dilution of R Culture.....	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷

0.1 cc. quantities of each of the above R and S dilutions were seeded together in blood broth and daily plates and subcultures made from the mixtures. It was obvious that in such experiments considerable variation in results would be obtained, but generally speaking, the cultures maintained the same relative proportions of R and S colonies for several transfers. Apparently the mere presence and growth of S cells in intimate association with R forms was not sufficient to cause the latter to revert to the S variety.

(2) R organisms were cultured in blood broth to which was added the bacteria from 100 cc. of homologous S culture, heated for 15 minutes at 60°C. Transfers were continued for fifteen subcultures without the appearance of S colonies. At the end of this time the virulence of the R culture was determined by mouse inoculation and found to be unchanged. Such experiments suggest that the R form is not able, under ordinary "*in vitro*" conditions, to utilize an S vaccine, as such, to build up its own polysaccharide.

(3) Moreover, under similar conditions of cultivation, when 10 per cent anti-R serum was added to the media, the change failed to occur; whereas, in the absence of the vaccine, reversion regularly took place after the appropriate number of transfers. Such a result can be explained by assuming that the vaccine, which contains, in addition to its type-specific antigen, the common group-specific protein (or R) antigen (8), absorbed the anti-R antibodies from the immune serum. In the absence of the anti-R antibodies the change failed to occur. This experiment, then, offers further proof of the rôle played by anti-R antibodies in effecting R→S reversion "*in vitro*."

(4) The possibility that the vaccine might be effective in producing reversion only in the presence of tissues under anaerobic conditions received some consideration. R forms were grown, under vaseline seal, in blood broth to which was added lymph-tissue, muscle tissue and ground up spleen, in addition to large amounts of vaccine. Subcultures were made daily for six days. No S colonies appeared at any time during the process and the cultures remained avirulent.

Thus, under the conditions employed, all attempts to produce the $R \rightarrow S$ change *in vitro* by the use of vaccines have uniformly failed.

The possibility that the vaccines became "digested" in the subcutaneous tissues of the mice, and that the R forms were able to utilize the "digestion" products to build up their own S substance also received attention. Attempts to reproduce such "digestion" "*in vitro*" will be considered in the subsequent paper.

DISCUSSION

The conversion of relatively avirulent pneumococci into highly virulent organisms is obviously a matter of considerable biological and epidemiological significance. Recent observations made in this laboratory suggest that R Pneumococci are not infrequently found in the flora of the upper respiratory tract of normal individuals. Such observations indicate that this form of the organism appears not only under artificial conditions of cultivation in the test-tube but may be considered as an evidence of biological adaptation to environment on the part of the bacteria. Moreover, the avirulent R form is potentially capable of again developing into the virulent S type under favorable circumstances. The factors determining such development in the human are difficult of analysis but under experimental conditions certain observations can be made in animals.

Attention is drawn first to the "*in vitro*" method of producing the $R \rightarrow S$ change,—the growth of R organisms in anti-R serum. In this connection the existence of anti-R antibodies in the sera of normal individuals, as shown in a previous paper (2), is believed to be a point of considerable significance, and may play a rôle in the $R \rightarrow S$ reversion process in the human being.

Griffith's observations on the conversion "*in vivo*" of avirulent R pneumococci into virulent, type-specific, S, organisms have been completely confirmed. In attempting an analysis of the causes responsible for reversion by the technique adopted by him certain points must be considered. He suggests that the mass of culture forms a nidus in which the attenuated pneumococci are protected from the bactericidal action of the tissues. But, as he himself indicates, this can play only a small part in the reversion process. Large amounts of R vaccine, amounts larger than those of the S vaccines

employed, as well as vaccines of other organisms, should also suffice to protect the R forms from phagocytosis and from the bactericidal action of the tissues. Nevertheless, reversion has never been effected under those conditions. Moreover, the finding of living R organisms in the subcutaneous tissues as late as twenty days after injection proves that opportunity to survive is not the only condition necessary to bring about the R→S change in the animal body. Other factors must play a rôle in the reversion process. Is it possible that the S vaccine, disintegrating in the animal tissues, supplies a suitable pabulum from which the living R organisms are able to resynthesize their own specific soluble substance?

A point of considerable difficulty in such a hypothesis is the explanation of the differences in results obtained by the use of Type I S vaccine heated at 60° and at 100°C. It is necessary to assume that Type I S vaccine, when heated at the higher temperature, becomes altered in such a way that it, or its disintegration products, can no longer be utilized by the R forms. But it has been shown (4) that heating at 100° in no way alters the specific carbohydrate fraction of the Type I *Pneumococcus*. This hypothesis, therefore, is inadequate unless it can be shown that the portion of the vaccine effective in causing reversion in some fraction other than the specific soluble substance as such.

The fact that large amounts of living R cultures by themselves produce the same effect as small amounts of live R cultures when the latter are injected together with S vaccines, suggests that the causes responsible for reversion under these two conditions must be closely related. In this connection it is of importance to point out that all the R strains used in these experiments yielded traces of the specific soluble substance of their original S type. This has been shown in this laboratory by Julianelle (9) in the case of the 1 R strain which offered the greatest resistance to reversion. It is reasonable, then, to assume that large amounts of living R cultures may yield a sufficient amount of S substance, (or the closely related substance necessary for reversion) from which the R forms may resynthesize their own specific polysaccharide. And apparently, once the process is initiated, it is carried on indefinitely, so long as the environment is suitable. However, on this basis, it remains difficult to explain why large amounts

of R vaccines fail to provide the necessary material; while relatively small amounts of living R cultures are able to supply this factor. It may be that the necessary substance is not present in sufficient quantities in the R vaccines; while it is elaborated in an adequate amount by the growth of the living R forms.

The failure of "*in vitro*" attempts to effect reversion by the aid of vaccines deserves some consideration. It was not found possible to induce the R \rightarrow S change by growing R forms in symbiosis with S organisms, or in media containing large amounts of S vaccines. Likewise, attempts to produce the change by growing R Pneumococci with S vaccines under partial anaerobiosis in the presence of animal tissues were unsuccessful. The failure to effect reversion by these procedures suggests that either the conditions, as provided, were not adequate, or that some factor must be provided by the animal body.

It has been suggested that the S vaccine may possess some "activating" or "co-ferment" property, which, working in conjunction with the synthesizing enzymes of the R form, enables the latter to build up its own S structure. If the effect of the S vaccine is due to such a property it apparently can exercise this function only in the presence of living tissues. Moreover, one is confronted with the difficulty of explaining how such a property becomes inactivated, in the case of Type I S vaccine, by heating at 100°C., and not in the cases of Type II S and Type III S vaccines similarly treated.

Another possibility is that the conditions created in the mouse by the injection of S vaccines may be the determining factors in inducing the R \rightarrow S change. It has been pointed out previously that all the R cultures used in these experiments yielded traces of specific soluble substance of their original S type. Nevertheless the animals were able to withstand the injection of comparatively large amounts of living R organisms by themselves. The mouse must therefore possess some capacity to overcome infection by organisms producing minimal amounts of S substance. It is possible that the injection of S vaccines may destroy or inhibit this limited ability of the mouse. Under such conditions the R forms may elaborate S substance in greater quantities, and as a consequence develop into S organisms.

In this connection attention is directed to the work of Sia (10). Employing serum-leucocyte mixtures in a specially constructed apparatus,

he reported the following observation. "The presence of a small amount of the purified soluble substance of the homologous type markedly altered the conditions in the mixtures so that even a small number of avirulent pneumococci were enabled to grow in the serum and leucocytes of animals which ordinarily possess the power to destroy such pneumococci in relatively large numbers."

However, in any of the explanations considered, it is impossible to account for the different effect produced by Type I vaccine heated at 60°C and at 100°C. The exact causes responsible for reversion, under these experimental conditions, therefore remain unexplained. Whatever they may be, the fact remains that when R cultures are injected in large amounts by themselves, or in small amounts together with the heat-killed vaccines of S organisms, the characteristics of the R organisms are actually altered. Comparable phenomena may play a rôle of great importance in many infectious processes. A focus of infection may be a point at which relatively harmless organisms assume virulent characteristics; for the subcutaneous injection, in white mice, of large amounts of avirulent pneumococci produces conditions quite analogous to those existing in a focus of infection.

SUMMARY

R forms of *Pneumococcus* may be converted into S forms of the homologous Type. In addition to the methods previously reported,—(1) animal passage and (2) growth in anti-R sera,—conversion may be effected by the following procedures as employed by Griffith; (1) The subcutaneous injection, in white mice, of large amounts of living R organisms. (2) The subcutaneous injection, in white mice, of small amounts of living R organisms together with the heat-killed bacteria from large amounts of homologous S cultures. There are "varying degrees of constancy of the R variant"; but by these means it has been possible to effect conversion of all R forms selected. Attempts to cause a further "degradation" of R organisms by continued growth in homologous immune serum have been unsuccessful.

Type II S and III S vaccines are equally effective in producing conversion when heated for 15' at 60°C., or for 15' at 100°C. Type I S vaccine, however, while effective in causing conversion when heated for 15' at 60°C., apparently loses this property when heated for 15' at 100°C.

R vaccines, and vaccines of other organisms, when injected together with live R cultures, have always failed to produce conversion.

The causes responsible for conversion under these experimental conditions are discussed and the possibility of the occurrence of a similar process under natural conditions in human beings is indicated.

CONCLUSIONS

1. R forms of *Pneumococcus* may be converted into S forms of the homologous type by the subcutaneous injection, in white mice, of large amounts of living R organisms.

2. R forms of *Pneumococcus* may similarly be converted into S forms of the homologous type by the subcutaneous injection, in white mice, of small amounts of living R organisms, together with the heat-killed bacteria from large amounts of S cultures.

3. By these methods Types II S and III S vaccines are equally effective in producing conversion when heated for 15' at 60°C., or for 15' at 100°C. Type I S vaccine is effective in producing conversion when heated for 15' at 60°C., but not when heated for 15' at 100°C.

4. R vaccines and the vaccines of other organisms are not effective in producing conversion.

5. All "*in vitro*" attempts to produce conversion by the use of vaccines have been unsuccessful.

6. The rôle which the phenomenon of conversion may play in infectious processes is indicated.

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THE TRANSFORMATION OF PNEUMOCOCCAL TYPES

II. THE INTERCONVERTIBILITY OF TYPE-SPECIFIC S PNEUMOCOCCI

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In the previous communication (1) it was shown that avirulent, non-type-specific, R forms of *Pneumococcus* could be converted into virulent, type-specific, S forms of the original type by the following procedures as employed by Griffith (2): 1) The subcutaneous injection, in white mice, of large amounts of living R forms: 2) The subcutaneous injection, in white mice of small amounts of living R forms together with the heat-killed bacteria from large quantities of the *homologous* S culture. By these procedures the R forms were invariably converted to S forms of the same specific type as that from which they had been originally derived.

Griffith further reported (2) that R forms of *Pneumococcus*, derived from S forms of any specific type, could be converted into type-specific organisms of *heterologous* S types by the following procedure:—the subcutaneous injection, in white mice, of small amounts of living R forms together with the heat-killed bacteria from large quantities of *heterologous* S cultures. In other words, he stated that it was possible to transform S *Pneumococci* of one specific type into other specific types through the intermediate stage of the R form. The present communication is concerned with the question of such transformation of *Pneumococcal* types.

Methods

The suspensions of heat-killed organisms were prepared in the same manner as described in the preceding paper (1). Similar controls were employed to eliminate the possibility of the existence of viable organisms in the vaccines.¹ In all critical experiments the number of control animals injected was equal to the number of

¹ The term vaccine indicates a suspension of heat-killed pneumococci.

test animals. The bacteria obtained from large quantities of culture, killed by heating, were also injected into animals together with cultures of other live organisms. In addition large amounts of vaccine were injected into animals intoxicated with alcohol. In no instance were viable pneumococci recovered from the controls. Notwithstanding the conclusive nature of the controls employed, even more convincing evidence as to the improbability of the existence of any viable organisms in the heat-killed suspensions will be offered in the subsequent description of the experiments.

The R strains which were used were derived in all instances from typical, type-specific, S pneumococci by growth in homologous immune serum. The possibility of the cultures containing a mixture of R forms, derived from S forms of more than one type, was eliminated in many experiments by the use of single-cell strains. In addition, proof of the nature of the R strains was obtained by converting them to the S form by growth in media containing anti-R serum (3). Under these conditions the R forms invariably reverted to the S form of the same specific type as that from which they were originally derived.

EXPERIMENTAL

Conversion of R Forms of Pneumococcus into S Forms of Heterologous Type by the Subcutaneous Injection, in White Mice, of Small Amounts of Living R Forms, Together with Large Amounts of Heat-Killed S Forms of Heterologous Type

In the course of early experiments it became apparent that the selection of the R strain played an important rôle in determining the results obtained. The particular 2 R culture (strain D/39/R) which was first chosen could be readily converted to the S form of the original type by all the methods which have been described: 1) animal passage; 2) growth in media containing anti-R serum; 3) the subcutaneous injection, in white mice, of large amounts of living R forms; 4) the subcutaneous injection, in white mice, of small amounts of living R forms together with the heat killed bacteria from large amounts of the *homologous* S culture.

In the first experiment twelve mice were injected with 0.25 cc. of living 2 R culture together with the bacteria from 90 cc. of a Type I S culture, heated for 15' at 60°C. All the mice died after an interval of 24 to 48 hours and typical Type II S pneumococci were recovered from the heart's blood of each animal. In all cases the R forms were converted to S forms of the same type as that from which they were originally derived.

Another 2 R culture, (strain N/D/39/R), was then obtained by growing a

typical Type II S pneumococcus in its homologous immune serum. After six transfers the culture was plated. An individual colony was then selected and transferred to blood broth. This 2 R culture could likewise be converted into S forms of the original type; but greater difficulty was experienced in bringing about the change than in the case of the 2 R culture previously employed. This second 2 R culture was injected into a series of twelve mice together with aliquot portions of the Type I S vaccine used in the preceding experiment. Quite different results were obtained. Ten of the twelve animals died after an interval of 24 to 48 hours and from the heart's blood of nine of these typical Type I S pneumococci were recovered. Cultures made from the heart's blood of the tenth mouse yielded only R forms. Two mice survived.

TABLE I

Conversion of R Forms of Pneumococcus into S Forms (1) of the Original Type (2) of the Type of the Vaccine

Type and amount of vaccine	Amount of living R culture	Number of mice	Result	Pneumococci recovered by culture	
				R and S forms	No. mice
Bacteria obtained from 90 cc. Type I S culture, heated at 60°C. for 15'.	Nil.	10	All survived; sacrificed 7 days.	All cultures negative.	10
ditto.	0.25 cc. 2 R (strain D/39/R)	12	All died 1-2 days.	Type II S	12
ditto.	0.25 cc. 2 R (strain N/D/39/R)	12	10 died 1-2 days. 2 survived; sacrificed 7 days.	Type I S R only. All cultures negative.	9 1

The results obtained by injecting these two different 2 R strains, together with the same Type I S vaccine, appear in Table I.

Abstract of Protocol.—

2 R culture, (strain D/39/R), + IS Vaccine, heated for 15' at 60°C.

Number of mice injected—12.

Number of mice died—12.

Reversion to S forms of the original type—12.

Reversion to S forms of the same type as the vaccine—0.

2 R culture, (strain N/D/39/R), + IS Vaccine, heated for 15' at 60°C.

Number of mice injected—12.

Number of mice died—10.

Reversion to S forms of the original type—0.

Reversion to S forms of the same type as the vaccine—9.

No reversion, only R organisms recovered—1.

Number of mice survived—2.

The Type I S organisms which were recovered from the heart's blood of the second series of mice possessed all the attributes of typical Type I S pneumococci. The cultures agglutinated specifically in Type I serum; they elaborated the specific soluble substance characteristic of Type I pneumococci, and were highly virulent for white mice. Subcultures made through twenty transfers retained the same properties and the organisms showed no tendency to revert to their original S type.

Since the same Type I S vaccine was used in the two experiments the variation in results obtained must have been referable to a difference in the R cultures employed. This difference was reflected in the greater difficulty experienced in causing the second 2 R culture to revert to the S form of its original type. Apparently in order that an R culture may revert to the S form of a heterologous type it must first be reduced to a definite stage in the "degradation" process.

This experiment also offers further proof of the absence of any viable organisms in the vaccine. It is highly improbable that the Type I S organisms recovered from the second series of mice could have developed from surviving forms in the vaccine; for no such forms were recovered from either the control mice or from the first series of mice which received the same vaccine.

An interesting result was obtained when still another 2 R culture was injected into mice along with Type I S vaccine. This third 2 R culture was obtained in the usual way by growing a typical Type II S pneumococcus in its homologous immune serum. When grown in anti-R serum, or injected subcutaneously in white mice in large amounts by itself, this R culture invariably reverted to the S form of the same type as that from which it was originally derived. Eleven mice were injected subcutaneously with 0.25 cc. of living R forms together with the bacteria from 100 cc. of Type I S culture heated for 15' at 60°C. Eight out of the twelve animals died after a period of one to two days, and Type I organisms were recovered from the heart's blood. In certain respects, however, the organisms recovered were not typical S forms. Although the cultures agglutinated specifically in Type I serum and produced the specific soluble substance characteristic of Type I pneumococcus, they did not possess maximal virulence for white mice nor

did they produce typical Smooth colonies. As a rule the cultures proved fatal to white mice in dilutions of 10^{-5} cc., occasionally in dilutions of 10^{-6} cc., but never in dilutions of 10^{-7} cc. The colonies were atypical in appearance, having neither the glossy, shiny surface of Smooth colonies nor the finely granular, ground-glass appearance of Rough colonies. For the most part the colonies were irregular in outline with nibbled margins. The outer zone was usually slightly rough; toward the center the majority presented a smooth wavy appearance; and in the middle of the colony there was frequently a crater-like depression. Other colonies more closely resembled typical R colonies and still others were suggestive of those of the S variety. That such colonies were not composed of a mixture of Rough and Smooth organisms was repeatedly proven by selecting individual colonies, subculturing, and plating for several transfers. Even when passed through mice colonies with the same characteristics were recovered from the heart's blood. These forms most probably represented incompletely developed S organisms partially "stabilized" at this phase of the reversion process. Such cultures could be converted into typical S forms by the subcutaneous injection in white mice of large amounts of the cultures alone.

In the course of the same experiment a 3 R culture, obtained by growing a typical Type III S pneumococcus in Type III serum, was injected into a series of eleven mice, together with aliquot portions of the same Type I S vaccine. Seven of the eleven animals succumbed and typical Type I S organisms were recovered from the heart's blood in each instance. All the colonies obtained from this series of animals were of the typical Smooth variety, and no "intermediate" forms were observed. All the cultures possessed maximal virulence for white mice.

Since the heat-killed suspension used in both series was the same the conclusion must be drawn that the atypical intermediate colonies recovered from the first series of mice developed from the 2 R culture and not from the vaccine. This observation offers still further proof of the absence of viable forms in the heat-killed suspensions.

In certain other experiments the injection of an R culture together with a heterologous S vaccine resulted in the recovery of a mixture of S forms from the animals. Such mixtures were composed exclusively of S organisms of the same type as the vaccine and the type from which the R forms had been originally derived. In all such cases S organisms were not obtained from any of the control mice. It must therefore be concluded that, in these instances, conditions were equally suitable for reversion of the R forms to the S forms of the original type, or to the type of the vaccine.

When a plate is composed of a mixture of colonies of various S types the Type III colonies are usually readily identified by their large size and clear, watery appearance. It is also possible, as a rule, to identify colonies composed of Type IS organisms. When examined against a dark background through a plate culture microscope Type I colonies are usually denser, more opaque and whiter than Type II colonies. Colonies composed of Type II and Group IV organisms, on the other

hand, are paler, more transparent and "watery." They also appear to undergo autolysis more readily than Type I colonies and frequently present a 'ring' or 'life-saver' appearance.

Experiments were next undertaken to determine whether one and the same R culture could be successively transformed into the S form of each of the specific types of pneumococcus.

A 2 R culture was obtained by growing a typical Type II pneumococcus in its homologous immune serum. After four transfers the culture was plated. A single R colony was selected and subcultured in blood broth. A single-cell strain was then obtained from this culture by the method of Avery and Leland (4). Four mice were injected with 0.25 cc. of the single-cell culture together with the bacteria from 100 cc. of a Type III S culture heated for 30' at 60°C. Three of the animals succumbed, and typical Type III S pneumococci, possessing all the characteristics of that type, were obtained from the heart's blood of each. One of these III S cultures was then converted into the R form by growth in Type III serum. After five transfers the culture was plated. A single R colony was selected and subcultured in blood broth. The resultant growth was plated and again a single colony was selected and subcultured. This process was repeated four times and the final culture was injected into four mice together with a Type I S vaccine heated for 30' at 60°C. All four mice died and typical Type I S organisms were recovered from the heart's blood in each instance. One of these typical Type I S cultures was again converted to the R form in the same manner as previously described. The resulting R culture was injected into four mice together with a vaccine prepared from a Group IV S culture. Two of the mice died yielding S organisms in the heart's blood. Cultures from the heart's blood of these two animals did not agglutinate specifically in Types I, II or III sera but were highly virulent for mice. Specific anti-serum was not available to test the agglutination of the Group IV S strains, but in view of the preceding results it was highly probable that the cultures were of the same variety as the Group IV vaccine.

During the various stages of this experiment whenever an R culture was obtained it was grown in media containing 10 per cent anti-R serum. In all cases, after a variable number of transfers, the R form was converted to the S form of that type from which it had last been derived. This observation lends support to the contention that in no instance was a mixture of R forms, derived from S forms of more than one type, present in the culture.

In summary, a typical Type II S pneumococcus was successively transformed, through the intermediate stage of the R form, into a Type III S pneumococcus, a Type I S pneumococcus, and a Group

TABLE II

The Effect of the Temperature at Which a Type I S Vaccine is Heated upon Its Efficacy in Inducing Transformation of Type

Type and amount of vaccine	Temp. at which vaccine was heated for 15'	Amount of living R culture	Number of mice	Result	Pneumococci recovered by culture	
					R and S forms	N mice
Bacteria obtained from 100 cc. Type I S culture	C.	Nil	4	All survived; sacrificed 8-10 days	All cultures negative	4
ditto	60°	0.25 cc. 2R	5	All died 1-2 days	Type I S	5
ditto	65°	Nil	2	Both survived; sacrificed 8-10 days	All cultures negative	2
ditto	65°	0.25 cc. 2R	5	All died 1-2 days	Type I S	5
ditto	70°	Nil	2	Both survived; sacrificed 8-10 days	All cultures negative	2
ditto	70°	0.25 cc. 2R	5	All died 1-2 days	Type I S	5
ditto	75°	Nil	2	Both survived; sacrificed 8-10 days	All cultures negative	2
ditto	75°	0.25 cc. 2R	5	All died 1-2 days	Type I S	5
ditto	80°	Nil	2	Both survived; sacrificed 8-10 days	All cultures negative	2
ditto	80°	0.25 cc. 2R	5	All died 1-2 days	Type I S	5
ditto	100°	Nil	2	Both survived; sacrificed 8-10 days	All cultures negative	2
ditto	100°	0.25 cc. 2R	5	Three died 1-2 days	R only	3
				Two survived; sacrificed 8-10 days	All cultures negative	2

IV S pneumococcus. At any stage of the cycle the R form could be converted to the S form of that type from which it had last been derived by growth in anti-R serum.

The Effect of the Temperature at Which an S Vaccine Is Heated upon Its Efficacy in Causing an R Culture, Derived from a Heterologous S Type² to Revert to the Type of the Vaccine

Griffith reported (2) that S vaccines, when heated at temperatures higher than 70°C., were rarely effective in causing R forms, derived from heterologous S types, to revert to the type of the vaccine. To verify this finding the following experiment was devised.

The bacteria obtained by centrifuging 4600 cc. of a Type I S culture were suspended in 23 cc. of plain broth. This suspension was divided into four equal portions of 3.5 cc. each, and two portions of 4.5 cc. each. (The larger portions were heated at the lower temperatures and a greater quantity of suspension was required for additional controls.) Each of these six portions of the same vaccine was heated for fifteen minutes at varying temperatures between 60°C. and 100°C., as outlined in the accompanying table. After heating, a volume of 2 cc. from each of the larger samples, and 1 cc. from each of the smaller samples was reserved for injection into control mice. To the remaining amounts of each portion of vaccine 1.25 cc. of a blood broth culture of a 2 R pneumococcus was then added. The quantities were so arranged that all mice, including the controls, received the heat-killed bacteria from 100 cc. of culture. In addition, each test animal received 0.25 cc. of living 2 R culture.

The results of the experiment appear in Table II.

Abstract of Protocol.—Type I S vaccine, heated for a period of 15' at temperatures between 60°C. and 80°C., when injected subcutaneously in white mice together with a living 2 R culture, apparently possesses the ability to cause the 2 R culture to revert to a Type I S pneumococcus. Type I S vaccine heated for 15' at 100°C. does not possess this property.

Attention should be called to certain features in this experiment. In the first place an unusually large number of transformations was obtained. Other experiments were not so uniformly successful. In another experiment a Type I S vaccine, heated for 15' at 70°C. caused a 2 R culture to revert to the type of the vaccine but did not do so when heated for 15' at 80°C. Again, in the experiment recorded above, the Type I S vaccine heated at 100°C. apparently had no effect on the 2 R culture. It did not even cause the R culture to revert to its original S type. In other experiments, however, a Type I S vaccine, heated for 15' at temperatures of 90° and 100°C., caused both 2 R and 3 R cultures to revert to their original S types. The results of such an experiment are reported in Table III.

² More correctly this should read "derived from the S form of a heterologous type." For the sake of brevity in this and succeeding instances the above expression has been adopted.

Abstract of Protocol.—In the experiment detailed in Table III Type I S vaccine, heated for 15' at 70°C., was effective in causing a 2 R culture to revert to a Type I S pneumococcus. When heated for the same period at a higher temperature than 70°C. Type I S vaccine was not effective in causing a 2 R culture to revert to the type of the vaccine. However, in several mice which received the Type I S vaccine heated at 80°, 90° and 100°C. the 2 R forms reverted to their original S type.

TABLE III

The Effect of Temperature at Which a Type I S Vaccine Is Heated upon Its Efficacy in Inducing Transformation of Type. (Second Experiment)

Type and amount of vaccine	Temp. at which vaccine was heated for 15'	Amount of living R culture	Number of mice	Result	Pneumococci recovered by culture	
					R and S forms	No. mice
Bacteria obtained from 80 cc. Type I S culture.	C.					
	60°	Nil	4	All survived; sacrificed 7 days	All cultures negative	4
ditto	60°	0.25 cc. 2 R	4	All died 1-2 days	Type I S R only	3 1
ditto	70°	Nil	2	Both survived; sacrificed 7 days	All cultures negative	2
ditto	70°	0.25 cc. 2 R	4	All died 1-2 days	Type I S R only	3 1
ditto	80°	Nil	2	Both survived; sacrificed 7 days	All cultures negative	2
ditto	80°	0.25 cc. 2 R	4	3 died 1-2 days; 1 survived	Type II S R only	2 1
ditto	90°	Nil	2	Both survived; sacrificed 7 days	All cultures negative	2
ditto	90°	0.25 cc. 2 R	4	All died 1-2 days	Type II S R only	2 2
ditto	100°	Nil	2	Both survived; sacrificed 7 days	All cultures negative	2
ditto	100°	0.25 cc. 2 R	4	3 died 1-2 days; 1 survived	Type II S R only	2 1

Similar results were obtained when a 3 R culture was injected into mice together with a Type I S vaccine heated at various temperatures. From the majority of the animals which received the Type I S vaccine heated at temperatures up to 80°C. S organisms of the same type as that of the vaccine were obtained. From the animals which received the Type I S vaccine heated at higher temperatures than

80°C. no Type I S organisms were recovered. Several of these mice, however, did yield typical Type III S pneumococci.

These findings may be summarized as follows:—Type I S vaccine, heated for 15' at various temperatures between 60° and 80°C. is effective in causing an R culture derived from a heterologous S type

TABLE IV

The Effect of the Temperature at Which Types II S and III S Vaccines Are Heated upon Their Efficacy in Inducing Transformation of Type

Type and amount of vaccine	Temp. at which vaccine was heated for 15'	Amount of living R culture	Number of mice	Result	Pneumococci recovered by culture	
					R and S forms	No. mice
Bacteria obtained from 90 cc. Type II S culture.	C.					
	60°	0.25 cc. 1 R	6	3 died 3 survived	Type II S	3
ditto	60°	0.25 cc. 3 R	8	All died	Type II S	8
ditto	100°	0.25 cc. 1 R	6	All survived		
ditto	100°	0.25 cc. 3 R	6	5 died 1 survived	Type III S	5
Bacteria obtained from 90 cc. Type III S culture.	60°	0.25 cc. 1 R	6	4 died 2 survived	Type III S	4
ditto	60°	0.25 cc. 2 R	6	4 died 2 survived	Type III S	4
ditto	100°	0.25 cc. 1 R	6	All survived		
ditto	100°	0.25 cc. 2 R	6	3 died 3 survived	Type II S	3

to revert to the type of the vaccine. When heated at temperatures higher than 80°C. Type I S vaccine does not cause an R culture derived from a heterologous S type to revert to the type of the vaccine; but frequently causes the R culture to revert to its original S type.

Experiments were then undertaken to determine whether vaccines prepared from S organisms other than Type I were subject to the same thermal differentiation as a Type I S vaccine.

(In the previous paper it was shown that vaccines prepared from cultures of Types II S and III S pneumococcus, whether heated at 60°C. or 100°C., were equally effective in causing R organisms, derived from the same S type as that of the vaccine, to revert to their original S type. It was also shown that vaccines prepared by heating cultures of Type I S pneumococcus at 60°C. were effective in causing a 1 R culture to revert to its original S type. However, when a Type I S vaccine was heated at 100°C. this property was destroyed and reversion failed to occur.)

Vaccines were prepared by heating a culture of Type II S pneumococcus at temperatures of 60°C. and 100°C. Equal portions of each lot of vaccine were injected into two series of mice together with 1 R and 3 R cultures, respectively. Similarly, vaccines were prepared by heating a culture of Type III S Pneumococcus at 60°C. and at 100°C. Two series of mice were injected with these vaccines together with 1 R and 2 R cultures, respectively.

The results of these experiments appear in Table IV.

From the foregoing results and from those obtained in previous experiments the following conclusions may be drawn:—

(1) Vaccines prepared by heating cultures of each of the three S types of pneumococcus at 60°C. are effective in causing R forms, derived from heterologous S types, to revert to the type of the vaccine.

(2) Vaccines prepared by heating cultures of each of the three S types at 100°C. are not effective in causing R forms, derived from heterologous S types, to revert to the type of the vaccine.

(3) Vaccines prepared by heating cultures of each of the three S types at 100°C. are frequently effective in causing 2 R and 3 R cultures to revert to the same specific type from which they were originally derived.

(4) Vaccines prepared by heating cultures of any S type, including Type I, at 100°C. are not effective in causing a 1 R culture to revert to its original S type.

The Effect of the Duration of Heating upon the Efficacy of a Type I S Vaccine in Causing an R Culture, Derived from a Heterologous S Type, to Revert to the Type of the Vaccine

Griffith reported that vaccines heated for long periods, even at temperatures as low as 60°C., were less effective than vaccines heated for short periods in causing R forms, derived from heterologous S types, to revert to the type of the vaccine. To test the effect of heating an S vaccine for varying periods the following experiment was arranged.

The bacteria obtained by centrifuging 4000 cc. of a Type I S culture were taken up in 20 cc. of plain broth and heated for 15' at 60°C. A quantity of 8 cc. of suspension was withdrawn and used in the first part of the experiment. The remaining 12 cc. of suspension were heated for a further period of 15' at the same temperature. Three cc. were then withdrawn and a like amount at half-hour intervals thereafter on three occasions. Five cc. of the first 8 cc. sample withdrawn were used for control purposes, a volume of 0.5 cc. being injected subcutaneously into each of ten mice. To each of the five 3 cc. samples 1.5 cc. of a living 2 R

TABLE V

The Effect of the Duration of Heating upon the Efficacy of a Type I S Vaccine in Inducing Transformation of Type

Type and amount of vaccine	Length of time during which vaccine was heated at 60°C.	Amount of living R culture	Number of mice	Result	Pneumococci recovered by culture	
					R and S forms	No. mice
Bacteria obtained from 100 cc. Type I S culture.	15'	Nil	10	All survived; sacrificed at intervals up to 17 days	All cultures negative	10
ditto	15'	0.25 cc. 2 R	6	5 died 1 survived	Type I S	5
ditto	30'	0.25 cc. 2 R	6	4 died 2 survived	Type I S	4
ditto	1 hour	0.25 cc. 2 R	6	5 died 1 survived	Type I S	5
ditto	1½ hours	0.25 cc. 2 R	6	4 died 2 survived	Type I S	4
ditto	2 hours	0.25 cc. 2 R	6	5 died 1 survived	Type I S	5

culture was added. Six test mice were inoculated in each part of the experiment with the heat-killed bacteria from 100 cc. of Type I S culture, together with 0.25 cc. of living 2 R culture.

The details of the experiment are given in Table V.

Abstract of Protocol.—Type I S vaccine, heated for as long a period as two hours at 60°C., when injected subcutaneously in white mice together with a living 2 R culture, apparently possesses the ability to cause the 2 R forms to revert to Type I S organisms. A Type I S vaccine heated for 2 hours at 60°C. is just as effective in producing reversion to the type of the vaccine as that heated for 15' at 60°C.

Mention should again be made of the fact that this also was an unusually successful lot of vaccine. In other experiments a smaller number of positive results was obtained and there appeared to be a slight falling off in the effectiveness of the vaccine when heated for prolonged periods at 60°C.

The Amount of Heat Killed S Organisms Necessary to Cause R Forms Derived from a Heterologous S Type, to Revert to the Type of the Vaccine

The minimal amount of vaccine capable of inducing transformation of type was then ascertained. An experiment was devised in which

TABLE VI

The Amount of Heat-Killed Suspension Necessary to Induce Transformation of Type

Amount of culture from which Type I S vaccine was prepared	Amount of living R culture	Number of mice	Result	Pneumococci recovered on culture	
				R and S forms	No. mice
100 cc.	0.25 cc. 2 R	4	4 died	Type I S	4
50 cc.	0.25 cc. 2 R	4	3 died 1 survived	Type I S	3
25 cc.	0.25 cc. 2 R	4	2 died 2 survived	Type I S	2
10 cc.	0.25 cc. 2 R	4	All survived	—	
100 cc.	Nil	4	All survived; sacrificed 7 days.	All cultures negative.	4

four series of mice received a living 2 R culture together with varying amounts of Type I S vaccine. In addition to the living R culture the first series of animals received the heat-killed bacteria from 100 cc. of S culture; the second from 50 cc.; the third from 25 cc. and the fourth from 10 cc. Four control mice received only the heat-killed bacteria from 100 cc. of S culture.

The results of this experiment appear in Table VI.

Abstract of Protocol.—From the heart's blood of all 4 mice which received the living 2 R culture together with the heat-killed bacteria from 100 cc. of Type I S culture, S organisms of the same type as the vaccine were obtained. Similarly

Type I S organisms were recovered from 3 out of 4 mice which received the heat-killed bacteria from 50 cc. and from 2 out of 4 which received the bacteria from 25 cc. From those which received the bacteria from 10 cc. no Type I S organisms were obtained. The control mice which were injected with the heat-killed bacteria from 100 cc. of Type I culture all survived. They were sacrificed at the end of 7 days and cultures from the blood and viscera were negative.

In many other experiments it has constantly been found that large amounts of vaccine are necessary to effect transformation of type by this procedure.

The Effect of Autolysis on the Efficacy of an S Vaccine in Causing R Forms, Derived from Heterologous S Types, to Revert to the Type of the Vaccine

Early in the work it was found that many lots of vaccine were relatively ineffective in causing R forms to revert to the Type of the vaccine. In searching for an explanation of these failures it was noticed that many mice in such unsuccessful experiments developed purpura to a marked degree. Julianelle and Reimann (5) have shown that the purpura-producing fraction of pneumococcus is released only during autolysis and is not present in heat-killed cultures in which autolysis has not taken place. This fact suggested a possible explanation for the ineffectiveness of certain lots of vaccine. It seemed possible that the cultures might have undergone partial autolysis before being heat-killed and, as a consequence, the vaccines made from such cultures were no longer effective in producing reversion. To test this hypothesis the following experiment was done:—

Cultures of Type I S and Type II S organisms were centrifuged and the deposit divided into two equal portions. One-half of the deposit from each culture was immediately heated at 60°C. for 15'; the other half was allowed to autolyze at 37°C. for 48 hours. At the end of this period the autolysate was subjected to a temperature of 60°C. for 15'. The two preparations were injected into a series of mice together with cultures of heterologous R organisms as detailed in Table VII.

Abstract of Protocol.—Autolysates of S cultures, when injected subcutaneously in white mice together with living R cultures, are not effective in causing R forms to revert, either to their original S type, or to the S type from which the autolysate was prepared. (Heat-killed suspensions of S organisms, however, kept in the ice-box for periods up to three weeks, have been found to be effective in causing R forms derived from heterologous S types, to revert to the type of the vaccine.)

This experiment offers evidence that it is not the specific soluble substance as such that is responsible for transformation of type in these procedures. The specific soluble substance of pneumococcus is not altered during autolysis and is present both in the autolysate and in the heat-killed suspension (6).

TABLE VII

The Effect of Autolysis on the Efficacy of an S Vaccine in Inducing Transformation of Type

Type and amount of vaccine	Type and amount of autolysate	Amount of living R culture	Number of mice	Result	Pneumococci recovered on culture	
					R and S forms	No. mice
Bacteria obtained from 80 cc. Type I S culture heated for 15' at 60°C.	Nil	0.25 cc. 2 R	4	4 died	Type I S	4
ditto	Nil	0.25 cc. 3 R	4	3 died 1 survived	Type I S	3
Nil	Bacteria obtained from 80 cc. Type I S culture allowed to autolyze for 48 hrs. at 37°C.	0.25 cc. 2 R	4	All survived; sacrificed 7 days.	All cultures negative	4
Nil	ditto	0.25 cc. 3 R	4	All survived; sacrificed 7 days.	All cultures negative	4

The Effect of Injecting S Cultures Killed by Other Agents than Heat Together with R Cultures Derived from Heterologous S Types

In the experiments reported up to this point the S cultures were invariably killed by heat.

In order to determine whether or not S organisms killed by other agents than heat possessed similar properties, cultures of S pneumococci were killed with the following substances:—formalin, iodine,

chloroform, tricresol, alcohol and acetone. None of the reagents selected destroy the specific soluble substance of pneumococcus (7). The smallest possible concentrations were employed to eliminate the toxic effect of the reagents themselves.

Six series of five mice each were injected with a living 2 R culture together with the bacteria from 100 cc. of Type I S culture which had been killed by adding minimal amounts of each of the above mentioned bactericidal substances. Reversion to the type of the vaccine did not occur in a single instance. At the same time five animals were injected with the bacteria from 100 cc. of the same Type I S culture, heated for 15' at 60°C. together with the same living 2 R culture. Type I S organisms were recovered from the heart's blood in each instance. Four control mice which received only the heat-killed vaccine survived.

The failure to induce reversion by the use of suspensions of S organisms killed by other agents than heat suggests one of two possibilities;—either that portion of the vaccine responsible for reversion may have been destroyed by the bactericidal substances; or, the toxic effect of the reagents may have created unfavorable conditions in the tissues of the animals.

Results Obtained by the Intraperitoneal Injection of Small Amounts of Living R Forms Together with the Heat-Killed Bacteria from Large Amounts of Heterologous S Cultures*

In all the preceding experiments living R forms and S vaccines were injected *subcutaneously* in white mice. Experiments were undertaken to determine whether similar transformations could be effected by the *intraperitoneal* injection of living R forms together with vaccines of S cultures. It was hoped that it would be possible during this procedure to follow the transformation process by withdrawing and examining portions of the peritoneal contents from time to time. The results were not uniformly satisfactory. In one experiment eight mice were injected intraperitoneally with a living 2 R culture together with a Type III S vaccine. Four of the animals died and Type III S organisms were recovered from the heart's blood. However, these organisms were only slightly agglutinable in Type III serum and the colonies were not of the large, typical Type III S variety. Typical Type III organisms were obtained by passing these cultures through second

mice. The first cultures were apparently composed of incompletely developed Type III S forms.

In another experiment varying amounts of a living 2 R culture were injected intraperitoneally together with large quantities of a Type I S vaccine. In no case did the R form revert to the type of the vaccine. It was therefore concluded that, while reversion to the type of the vaccine could be effected intraperitoneally, the subcutaneous route was the method of choice.

Attempts to Determine the Minimal Time Required to Effect Reversion of R Forms into Organisms of Heterologous S Type

In different experiments there was a great variation in the interval required to effect reversion. The shortest time in which an animal succumbed, yielding S organisms of the same type as that of the vaccine, was eighteen hours; the longest nine days. In some experiments virulent S organisms were recovered at the site of injection from apparently healthy animals which were sacrificed at the end of seven days. The finding of virulent bacteria in local abscesses in otherwise healthy animals after such an interval suggested that the mice had acquired considerable general immunity before the R forms had had the opportunity to develop into the S form. In the most successful experiments the usual time at which the animals succumbed was from one and one-half to two days after injection. However, if, in this period, S forms had developed in sufficient numbers to overwhelm the animal the actual time necessary for the reversion process to occur at the site of injection was probably much shorter. An attempt was made to determine the minimal time required in the following way.

A series of mice was injected subcutaneously with an S vaccine. Thereafter, at intervals of two, four, eight, twelve, and twenty-four hours living R forms were introduced into the same animals at the same location. Reversion to the type of the vaccine occurred in a moderate number of animals which received the living R forms as late as eight hours after the injection of the vaccine. Reversion did not occur in any animal which received the living culture after an interval longer than eight hours. However, only a small number of animals was employed in this experiment and for this reason the results cannot be considered conclusive. The procedure of introducing the living R forms simultaneously with the vaccines uniformly gave a higher proportion of positive results.

Attempts to Effect Reversion by the Injection of Living R Forms and S Vaccines in Different Locations in the Same Animals

Various ways in which S vaccines might act in causing reversion of R forms in the animal body were considered. One of the possibilities was that the general conditions created in the animal by the injection of such large amounts of vaccine might be suitable for the development of organisms of the S variety. If the reversion process depended upon the existence of such general conditions it might be possible to produce a comparable state by the injection of S vaccines and living R forms into different locations in the same animals. Six mice were injected with a Type I S vaccine in one inguinal region. Simultaneously a living 2 R culture was injected into the opposite inguinal region. One of the six mice died in two days and organisms of the same type as the vaccine were recovered from the heart's blood. The other five mice survived. However, such experiments are not conclusive for it is always possible that no matter where the living R forms are injected some organisms may find their way to the site of the vaccine. Because of this difficulty no further experiments were attempted along these lines.

Attempts to Effect Reversion by the Injection of Living R Forms of Pneumococcus Together with S Vaccines of Friedländer's Bacillus

It has been demonstrated in this laboratory that Type II pneumococcus and Type B Friedländer's bacillus elaborate specific soluble substances which are chemically and immunologically similar, although not identical (8). Experiments were therefore undertaken to determine whether it was possible to convert R pneumococci, derived from other S types than Type II, into Type II S organisms, by the use of a Friedländer Type B vaccine. R forms of pneumococci, derived from both Type I S and Type III S organisms, were injected into a series of mice together with a Type B Friedländer vaccine. The experiments were accompanied by certain difficulties, for it was found that the primary toxicity of S Friedländer vaccine was considerable.

In the first experiment a series of four mice was injected with the bacteria from 80 cc. of Type B Friedländer culture along with a living culture of 3 R pneumococcus. A similar number of animals was injected with the same Friedländer

vaccine and a culture of 1 R pneumococci. All the animals died after an interval of less than twenty-four hours but only R forms of pneumococci were recovered from the heart's blood.

The converse of the above experiment was then done.

R forms of Friedländer bacilli were injected into mice along with the heat-killed bacteria from a Type II S pneumococcus culture. R forms derived from each of the three specific types A, B, C, and from a strain of the heterogeneous group X were employed. Four mice were injected in each experiment. Two animals died after a period of twelve hours; the remainder survived and were sacrificed after a period of six days. Cultures made from the site of injection and from the heart's blood did not yield S Friedländer bacilli in a single instance. R forms were found at the site of injection in a large proportion of cases. Cultures of the recovered R forms were passed through a second series of mice and the virulence of the organisms was found to remain unchanged. Unfortunately many of the mice in this experiment developed ulcers at the place of injection. It is possible that this fact may have had some effect in determining the results obtained. It is also possible that the particular R strains of Friedländer's bacillus which were selected were not suitable for reversion.

Attempts to Convert R Forms of Pneumococci into Organisms of the Heterologous S Type by in Vitro Methods

The *in vitro* methods which were employed in attempts to convert R forms into organisms of the homologous S type, by the use of vaccines, have been described in a preceding paper (1). All the procedures adopted gave negative results. Similar attempts were made to convert R forms into S organisms of heterologous types. All such experiments were unsuccessful. It must therefore be concluded that, either the *in vitro* conditions, as provided, were inadequate, or that living tissues are essential for the reversion process. An attempt to partially reproduce *in vivo* conditions in the test tube was made in the following way.

Large quantities of a Type I S vaccine were injected intraperitoneally into five mice. The animals were sacrificed at intervals of 2, 4, 8, 12 and 24 hours and the peritoneal contents washed out with plain broth. The washings were transferred to test tubes and seeded with a 2 R culture. Plates made from the resulting growth, however, yielded only R colonies and the cultures remained avirulent for white mice.

The negative results of this experiment suggest that living tissues may play a part in the reversion process. However, it should be pointed out that in previous experiments the intraperitoneal route was not found as suitable as the subcutaneous route. The possibility remains that if the conditions obtaining in the subcutaneous tissues of the mouse could be reproduced in the test tube transformation of type might be effected *in vitro*.

Attempts to Convert S Pneumococci of One Specific Type Directly into S Organisms of Another Specific Type

In all the experiments described in which pneumococci have been converted from one specific S type into other specific S types the transformation has been effected through the intermediate stage of the R form. Inasmuch as the phenomenon of transformation of type had never been observed in type-specific S cultures under artificial cultivation it seemed most unlikely that S organisms could be transformed directly from one specific type to another specific type. An attempt to effect such a direct transformation of type was made in the following manner. Mice were injected subcutaneously with the smallest possible dilutions of living S cultures together with the heat-killed bacteria from large amounts of heterologous S cultures. For example a Type II S culture, in dilutions of 10^{-7} , 10^{-8} , and 10^{-9} cc. was injected into a series of mice together with a Type I S vaccine. In all cases the animals succumbed and only S organisms of the same type as those introduced in the living cultures were recovered from the heart's blood. Direct transformation from one type to another did not occur in a single instance. These experiments also prove that large quantities of vaccine have no inhibitory effect on any viable forms introduced with the vaccine. On the contrary the animals that received both the living culture and the vaccine succumbed in a much shorter period of time than those which received only the dilutions of living culture.

DISCUSSION

The transformation of pneumococci from one specific type into other specific types is a phenomenon of wide bacteriological and epidemiological significance. It has not been conclusively demonstrated

that transformation of type actually occurs under natural conditions. Griffith (2) has presented certain evidence which indicates the possibility of such an occurrence during disease processes, but further work is required to establish definitely the validity of these observations. In any case the demonstration that transformation of type may be effected experimentally shows that the various types of pneumococcus are closely related biologically. Indeed it is possible to think that these various types may represent attempts on the part of the organism to adapt itself to varying environmental conditions.

It is important to note that it was found impossible to transform S organisms directly from one specific type into other specific types. Change of type was invariably brought about through the intermediate stage of the R form. The R form of the organism is most readily produced by growing S organisms in their homologous immune serum. It may also be produced by subjecting S pneumococci to unfavorable environmental conditions,—such as, growth in poor media, growth at temperatures between 40° and 42°C., and growth in media containing small traces of bile. The R form, therefore, probably results from attempts of S bacteria to adapt themselves to unfavorable environmental conditions. Once reduced to the R state the organisms potentially have the capacity to develop the S structure of any of the various specific S types. They most readily assume the characteristics of that S type from which they were last derived; but under the influence of certain conditions they may also develop the S structure of other specific types.

What conditions determine the development of S characteristics? The change may be induced experimentally by subcutaneously injecting, in white mice, large amounts of an S vaccine together with living R forms. The type of S structure which the R forms assume under these conditions is apparently dictated by two circumstances; (1) The degree of "degradation" to which the R forms have been subjected; (2) The degree of heat to which the S vaccine has been exposed. If the R forms have been reduced to a definite state in the "degradation" process they assume the characteristics of the same S type as the vaccine. If the R forms have been only partially "degraded" they assume the characteristics of that S type from which they were originally derived. Similarly, if the vaccine is heated at a tempera-

ture between 60° and 80°C. the R forms revert to the type of the vaccine: if the vaccine is heated at a temperature higher than 80°C. the R forms revert to the S type from which they were originally derived.

What are the causes responsible for transformation of type as induced by this procedure? In the previous paper various possibilities were considered to explain the way in which S vaccines might act in causing R forms, derived from the same S type as the vaccine, to revert to their original S type. It was pointed out that the precise factor responsible for reversion, as brought about by this procedure, was not understood. If the causes determining reversion of R forms to their original S type are not understood it is even more difficult to interpret the conditions under which R forms assume the characteristics of S organisms of the same type as the vaccine. That property of the vaccine responsible for reversion does not exactly correspond with any known substance or property of S organisms. It cannot be the S substance itself, for it has been shown that the carbohydrate fraction of pneumococcus is not altered by heating at 100°C. (9), and its specificity is not destroyed during autolysis. Moreover, the efficacy of an S vaccine in inducing transformation of type does not parallel the antigenic properties of the vaccine. It has been shown that vaccines of S pneumococci are equally good antigens whether heated at 60°C. or at 100°C.

It is possible that S vaccines may exert their effect in one of two ways:—(1) Directly on the R forms themselves; (2) On the tissues of the animals in which they are injected.

The failure of all *in vitro* attempts to secure transformation of type suggests that, if the vaccine exerts its influence directly on the living R forms, it does so only under very precise conditions. If it were possible to reproduce in the test tube the conditions obtaining in the subcutaneous tissues of the mouse, transformation of type might be effected *in vitro*. However, it would be difficult to duplicate experimentally the conditions created by the disintegration and digestion of large amounts of vaccine in the living tissues of an animal.

A second possibility is that the conditions created in the subcutaneous tissues of the mouse offer a suitable environment in which the R forms may build up their S structure. In the previous paper it was pointed out that, under natural conditions, the white mouse "possessed

some capacity to overcome infection by organisms producing minimal amounts of S substance." It was further suggested that "the injection of S vaccines might destroy or inhibit this limited ability of the mouse and under such conditions the R forms might develop into S organisms." May it not be possible that the injection of an S vaccine only inhibits or destroys the capacity of the mouse to overcome infection by that particular S type? Under such circumstances may not the R organism, potentially capable of synthesizing any type of polysaccharide, be able to elaborate that particular S substance most suitable for the survival of the organism in its environment?

In this connection reference is again made to the work of Sia (10). Employing serum-leucocyte mixtures in a specially constructed apparatus, he reported the following observation.

"The presence of a small amount of the purified soluble substance of the homologous type markedly altered the conditions in the mixtures so that even a small number of avirulent pneumococci were enabled to grow in the serum and leucocytes of animals which ordinarily possess the power to destroy such pneumococci in relatively large numbers." Sia further reported that this effect was highly type-specific for "a Type II substance assisted the growth of only pneumococcus Type II; likewise a Type III substance, the growth of pneumococcus Type III only."

Any such explanation, however, fails to account for the different effects produced by vaccines heated at temperatures above and below 80°C. Further work is therefore required to understand clearly the causes responsible for transformation of type as induced by Griffith's technique.

In the previous paper it was pointed out that R forms of pneumococcus could be found in the flora of the upper respiratory tract of many normal individuals. It was suggested that these forms resulted from attempts of the bacteria to adapt themselves to unfavorable environmental conditions. Although degraded to the R form these organisms still retained the capacity of again developing into virulent, type-specific, S pneumococci. Any such development would appear to be dictated by conditions in the environment. Those environmental conditions would also determine the particular S type which the R organisms may assume. In the absence of more precise data concerning such transformations further speculation is unprofitable. However, the possibilities of alteration in type under natural and

disease conditions cannot be ignored and may attain proportions of much significance in infectious and epidemiological problems.

CONCLUSIONS

1. Type-specific S pneumococci may be transformed from one specific S type into other specific S types through the intermediate stage of the R form.

2. R forms of pneumococci, derived from any specific S type, may be transformed into S organisms of other specific types by the following procedure:—The subcutaneous injection, in white mice, of small amounts of living R forms together with vaccines of heterologous S cultures.

(i) S vaccines heated for 15' at temperatures between 60° and 80°C., are effective in causing R forms, derived from heterologous S types, to revert to the type of the vaccine.

(ii) S vaccines heated for 15' at temperatures between 80° and 100°C., are not effective in causing R forms, derived from heterologous S types, to revert to the type of the vaccine.

(iii) S vaccines heated for 15' at temperatures between 80° and 100°C., may cause 2 R and 3 R cultures to revert to their original S type.

(iv) S vaccines of any type, including Type I, heated for 15' at temperatures between 80° and 100°C., are not effective in causing 1 R cultures to revert to their original S type.

(v) S vaccines heated for periods as long as two hours at 60°C. are effective in causing R forms, derived from heterologous types, to revert to the type of the vaccine.

3. A single cell R strain, derived from a Type II S pneumococcus, has been successively transformed into a Type III S, a Type I S and a Group IV S culture.

4. Corresponding with the various degrees of "degradation" of the R form there are varying degrees of "development" of the S form.

5. The nature of the conditions responsible for alteration of type as induced by these procedures has been investigated and the causes responsible for the transformations are discussed.

6. All attempts to produce transformation of type *in vitro* have been unsuccessful.

7. The rôle which the phenomenon of transformation of type may play in problems of infection and epidemiology is indicated.

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